Purification and characterization of a novel phosphoenolpyruvate carboxylase from banana fruit

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Phosphoenolpyruvate carboxylase (PEPC) from ripened banana (*Musa cavendishii* L.) fruits has been purified 127-fold to apparent homogeneity and a final specific activity of 32 μmol of oxaloacetate produced/min per mg of protein. Non-denaturing PAGE of the final preparation resolved a single protein-staining band that co-migrated with PEPC activity. Polypeptides of 103 (α-subunit) and 100 (β-subunit) kDa, which stain for protein with equal intensity and cross-react strongly with anti-(maize leaf PEPC) immune serum, were observed following SDS/PAGE of the final preparation. CNBr cleavage patterns of the two subunits were similar, but not identical, suggesting that these polypeptides are related, but distinct, proteins. The enzyme's native molecular mass was estimated to be about 425 kDa. These data indicate that in contrast to the homotetrameric PEPC from most other sources, the banana fruit enzyme exists as an $\alpha_2\beta_2$ heterotetramer. Monospecific rabbit anti-(banana PEPC) immune serum effectively immunoprecipitated the activity of the purified enzyme. Immunoblotting studies established that the 100 kDa subunit did not arise via proteolysis of the 103 kDa subunit after tissue extraction, and that the subunit composition of banana PEPC remains uniform throughout the ripening process. PEPC displayed a typical pH activity profile with an alkaline optimum and activity rapidly decreasing below pH 7.0. Enzymic activity was absolutely dependent on the presence of a bivalent metal cation, with Mg^{2+} or Mn^{2+} fulfilling this requirement. The response of the PEPC activity to PEP concentration and to various effectors was greatly influenced by pH and glycerol addition to the assay. The enzyme was activated by hexose-monophosphates and potently inhibited by malate, succinate, aspartate and glutamate at pH 7.0, whereas the effect of these metabolites was considerably diminished or completely abolished at pH 8.0. The significance of metabolite regulation of PEPC is discussed in relation to possible functions of this enzyme in banana fruit metabolism.

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC; orthophosphate:oxaloacetate carboxy-lyase [phosphorylating], EC 4.1.1.31) is a ubiquitous plant cytosolic enzyme that catalyses the irreversible β-carboxylation of PEP to yield oxaloacetate and P_i. This enzyme is particularly abundant in the mesophyll cells of C_4 and CAM leaves where it participates in photosynthesis by catalysing the initial fixation of atmospheric CO_2. The physical and kinetic/regulatory properties of C_4- and CAM-leaf PEPCs have been examined in some detail [1–4]. Both allosteric mechanisms and reversible protein kinase-mediated phosphorylation are believed to be of critical importance in the *in vivo* regulation of PEPC in C_4 and CAM leaves [1–4]. In contrast, the properties and functions for the enzyme from C_3 plants and non-photosynthetic tissues of C_4 and CAM plants are less well understood. Proposed roles for the C_3 enzyme are diverse and include: (i) regulation of cellular pH and cation balance [5]; (ii) production of dicarboxylic acids used as respiratory substrates by bacteroids of legume root nodules [6]; (iii) the anaplerotic replenishment of Krebs' cycle intermediates consumed in biosynthesis [5–8]; and (iv) providing both an ADP-independent 'bypass' to cytosolic pyruvate kinase (PK_c) and P_i-recycling during nutritional P_i deprivation [9].

Many climacteric fruit store imported photosynthate in the form of starch in amyloplasts and as ripening proceeds this carbon is exported into the cytosol and converted into sugars and/or CO_2. This process is applicable to the banana fruit in which starch constitutes about 20% of the fresh weight of mature unripe fruit and is almost quantitatively converted into soluble sugars during ripening with about 2 to 5% lost as CO_2 in respiration [10,11]. Hence, the banana fruit, by virtue of the rapid and highly predictable manner with which it progresses through changes in carbon allocation during ripening, provides an excellent model system for the study of the regulation of glycolytic and gluconeogenic carbon flux in higher plants. Many of the mechanisms of fine control, however, need to be elucidated. Two groups have demonstrated that decreases and increases in the concentrations of phosphoenolpyruvate (PEP) and pyruvate, respectively, are associated with the onset of the marked and rapid elevation in post-harvest CO_2 release (or 'respiratory climacteric') of ripening banana fruit [12,13]. This positive crossover suggests that activation of the PEP-metabolizing cytosolic enzymes PK_c and/or PEPC may be the initial response of glycolysis at the climacteric. This notion is consistent with various reports indicating that primary and secondary control of plant cytosolic glycolytic flux from hexose-phosphate to pyruvate is at the level of PEP and fructose 6-phosphate (Fru 6-*P*) utilization respectively [12–15].

The aim of the present study was to purify and characterize the physical, immunological and kinetic properties of banana fruit PEPC as a first step towards the formulation of a model for the *in vivo* regulation of PEP utilization in this tissue. Our results indicate that the banana fruit PEPC possesses a novel subunit composition and is potently regulated by several intermediates of carbohydrate and nitrogen metabolism in a pH-dependent fashion.

Abbreviations used: Glc 1-*P*, glucose 1-phosphate; Glc 6-*P*, glucose 6-phosphate; Fru 6-*P*, fructose 6-phosphate; Gly 3-*P*; glycerol 3-phosphate; PEP, phosphoenolpyruvate; DTT, dithiothreitol; PEPC, PEP carboxylase; PK, pyruvate kinase; PK_c, cytosolic pyruvate kinase.

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EXPERIMENTAL

Chemicals and plant material

Hepes, PEP, bis-tris-propane, L-malic acid, Coomassie Blue R-250 and dithiothreitol (DTT) were from Research Organics Inc. (Cleveland, OH, U.S.A.). NADH was from Boehringer Mannheim (Montreal, Que., Canada). Tris base, SDS and ammonium persulphate were from Schwarz/Mann Biotech (Cambridge, MA, U.S.A.). Acrylamide was from Sangon Co. (Scarborough, ON, Canada), whereas bisacrylamide was from Bio-Rad (Toronto, ON, Canada). Ribi adjuvant (product code R730) was obtained from Ribi Immunochemical Research (Hamilton, MT, U.S.A.). All other fine biochemicals, coupling enzymes, SDS and native PAGE molecular-mass standards, alkaline phosphatase-tagged goat anti-(rabbit IgG) IgG, Q-Sepharose and Fast Violet B were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DE-52 was from Whatman (Hillsboro, OR, U.S.A.). Poly(vinylidene difluoride) membranes (Immobilon transfer; 0.45 μm pore size), a Shodex KW-804 gel-filtration HPLC column, and a Protein-Pak Phenyl-5PW (8 mm × 75 mm) column were from Millipore-Waters (Mississauga, ON, Canada). Superose-6 Prep Grade and an FPLC system were purchased from Pharmacia (Baie d'Urfe, PQ, Canada). All other reagents were of analytical grade and were purchased from BDH Chemicals (Toronto, ON, Canada). Anti-(maize leaf PEPC) immune serum was a kind gift of Dr. Raymond Chollet (University of Nebraska, Lincoln, NE, U.S.A.). All buffers were degassed and adjusted to their respective pH values at 25 °C.

For enzyme purification, ripe banana (*Musa cavendishii* L. cv. Cavendish) fruit were purchased from a local retailer and used the same day. For the time-course studies, mature, green banana fruit were obtained from a local fruit wholesaler prior to treatment with ethylene gas. Hands were placed in a sealed chamber and treated at 25 °C in the dark with 1000 p.p.m. ethylene in air as described by Hubbard and co-workers [16].

Enzyme assays

The PEPC and puruvate kinase (PK) reactions were routinely coupled to the lactate dehydrogenase and malate dehydrogenase reactions, respectively, and assayed at 30 °C by monitoring NADH oxidation at 340 nm using a Gilford 260 recording spectrophotometer. Coupling enzymes were desalted before use. All assays were: (i) initiated by the addition of enzyme preparation; (ii) corrected for NADH oxidase activity; and (iii) linear with respect to concentration of enzyme assayed. The reaction mixture for PK was as described by Ball and co-workers [13]. PK assays were corrected for interference by PEP phosphatase activity by omitting ADP from the reaction mixture. Standard assay conditions for PEPC were: 50 mM Hepes/NaOH (pH 8.0), 1.5 mM PEP, 2.5 mM $KHCO_3$, 12 mM $MgCl_2$, 0.15 mM NADH and 2 units of rabbit muscle malate dehydrogenase in a final volume of 1.0 ml. One unit of enzyme activity is defined as the amount of enzyme resulting in the production of 1 μmol of product/min at 30 °C.

Kinetic studies

A Varian DMS 200 spectrophotometer was used for all kinetic studies. Apparent K_m values were calculated from the Michaelis–Menten equation, whereas $S_{0.5}$ and h (Hill coefficient) values were calculated from the Hill equation. Both equations were fitted to a non-linear least-squares regression computer kinetics program [17]. K_a and I_{50} values (concentrations of activator and inhibitor producing 50% activation and inhibition of enzyme activity, respectively) were determined using the aforementioned computer kinetics program [17]. PEPC's $K_m(HCO_3^-)$ was determined according to the method of Bauwe [18]. All kinetic parameters are the means of at least two determinations.

Preparation of clarified homogenates used in time-course studies

Peeled banana fruit at various stages of ripeness (i.e. 0, 2, 4, 6, 8 and 11 days after ethylene treatment of green preclimacteric bananas) was ground under liquid N_2 and stored at −80 °C until used. Frozen tissue was homogenized (1:8; w/v) using a mortar and pestle and a small scoop of sand in ice-cold 50 mM Hepes/NaOH (pH 7.8) containing 1 mM EDTA, 50 mM NaF, 2.5 mM DTT, 10 mM thiourea, 2% (w/v) poly(ethylene glycol) 8,000, 1 mM PMSF and 2.5% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was centrifuged at 16000 *g* for 10 min at 4 °C, and the resulting supernatant assayed for PK and PEPC activity as described above.

Buffers used in banana PEPC purification

Buffer A: 50 mM Tris base (pH unadjusted) containing 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 2 mM L-malate, 10% (v/v) glycerol, 10 mM thiourea, 2.5 mM $MgCl_2$, 2% (w/v) poly(ethylene glycol) 8000, 0.1% (v/v) Triton X-100, 1.5% (w/v) insoluble polyvinylpolypyrrolidone, 2 mM PMSF and 1 mM DTT. Buffer B: 50 mM Tris/HCl (pH 7.3) containing 2 mM L-malate, 10% (v/v) glycerol, 2.5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT. Buffer C: 200 mM potassium phosphate (pH 6.5) containing 10% (v/v) glycerol, 2 mM L-malate, 2.5 mM $MgCl_2$ and 1 mM DTT. Buffer D: 10 mM potassium phosphate (pH 7.5) containing 20% (v/v) glycerol, 5 mM L-malate, 0.5 mM EDTA, 2.5 mM $MgCl_2$ and 1 mM DTT. Buffer E: 50 mM potassium phosphate (pH 7.0) containing 30% (satd.) $(NH_4)_2SO_4$, 10% (v/v) glycerol, 0.1 mM EDTA and 1 mM DTT. Buffer F: 15 mM potassium phosphate (pH 7.0) containing 30% (v/v) ethylene glycol, 0.5 mM EDTA and 1 mM DTT. Buffer G: 50 mM Mes/NaOH (pH 6.8) containing 500 mM NaCl. Buffer H: 150 mM potassium phosphate (pH 7.0) containing 5% (v/v) glycerol, 1 mM EDTA and 1 mM DTT.

Purification of banana fruit PEPC

All procedures were carried out at 0 to 4 °C unless otherwise noted.

Crude extract

Peeled and diced banana fruit (900 g) was homogenized in 1.35 l of buffer A using a Waring blender and a Polytron. The homogenate was centrifuged at 14300 *g* for 15 min and the supernatant filtered through two layers of cheesecloth and one layer of Miracloth.

Batchwise DE-52

The supernatant was gently stirred for 30 min in the presence of 200 g (wet wt.) of DE-52 which had been pre-equilibrated in buffer B and collected with suction on a Buchner funnel. The mixture was allowed to stand for a further 30 min, and the matrix was isolated by filtration on a Buchner funnel. The filtrate was treated with DE-52 a further two times as described above. The combined DE-52 resin was washed with 750 ml of buffer B by filtration through the Buchner funnel with suction. Absorbed proteins were eluted by stirring the washed matrix for 10 min with 300 ml of buffer C, followed by filtration on the Buchner funnel as described above. This process was repeated twice and

the combined filtrates were pooled, adjusted to pH 7.3 with 5 M NaOH, and concentrated to 225 ml using a Millipore Minitan ultrafiltration system fitted with six 100000 NMWL cellulose plates.

Q-Sepharose chromatography

The concentrated DE-52 eluate was diluted 3-fold in water containing 1 mM DTT and loaded at 3 ml/min on to a column of Q-Sepharose (1.6 cm × 22 cm) pre-equilibrated in buffer D. The column was connected to an FPLC system, washed with buffer D until the A_{280} decreased to approximately 0.1, and then with buffer D containing 75 mM potassium phosphate (fraction size 10 ml). The enzyme was eluted in a sharp peak following a step from 75 to 200 mM potassium phosphate. Peak activity fractions were pooled and concentrated to about 13 ml using an Amicon PM-30 ultrafilter.

Protein Pak Phenyl-5PW FPLC

A solution of $(NH_4)_2SO_4$ (100% saturation; pH 7.0) was added to the concentrated Q-Sepharose fractions to bring the final $(NH_4)_2SO_4$ concentration to 30% (saturation). The solution was stirred for 20 min and centrifuged for 10 min at 16000 *g*. The supernatant was absorbed at 0.5 ml/min on to a Protein Pak Phenyl-5PW column (0.8 cm × 7.5 cm) that had been connected to the FPLC system and pre-equilibrated with buffer E (fraction size 1.75 ml). The column was washed with buffer E until the A_{280} decreased to baseline levels, and then eluted in a stepwise fashion using decreasing concentrations of buffer E and simultaneously increasing concentrations of buffer F. The enzyme eluted in a sharp peak following the step from 40% to 70% buffer F (60% to 30% buffer E). The pooled peak fractions were concentrated 5-fold with an Amicon YM-30 ultrafilter, then diluted 5-fold with buffer G. This was followed by another cycle of concentration and dilution as just described.

Chelating Sepharose 6B chromatography

The Phenyl-5PW pooled peak fractions were absorbed at 1 ml/min and room temperature on to a column of chelating Sepharose (1 cm × 4.3 cm) which had been charged with 50 mM $FeCl_2$ and pre-equilibrated with buffer G (fraction size 2.5 ml). The column was washed with buffer G until the A_{280} decreased to baseline and the enzyme eluted with 50 mM Pipes/NaOH (pH 7.5) containing 500 mM NaCl. The pooled peak fractions were adjusted to contain 10% (v/v) glycerol, 2 mM $MgCl_2$ and 1 mM DTT, and concentrated to 1 ml using an Amicon YM-30 ultrafilter.

Superose 6 gel-filtration FPLC

The concentrated pooled fractions from the chelating Sepharose column were applied at 0.1 ml/min on to a column (1.6 cm × 50 cm) of Superose 6 Prep grade that had been attached to the FPLC system and pre-equilibrated with buffer H (fraction size 1.5 ml). Pooled peak fractions were concentrated as above to 1.5 ml, divided into 50 μl aliquots, frozen in liquid N_2 and stored at −80 °C. The purified enzyme was stable for at least 6 months when stored frozen.

Antibody production

After collection of preimmune serum, purified PEPC [500 μg, dialysed overnight against PBS (20 mM potassium phosphate, pH 7.4, containing 150 mM NaCl)] emulsified in Ribi adjuvant (total volume 1.0 ml) was injected (700 μl subcutaneously, 300 μl intramuscularly) into a 2 kg New Zealand rabbit. Booster injections (250 μg) of the same protein emulsified in Ribi adjuvant were administered subcutaneously at 4 and 5 weeks. Ten days after the final injection blood was collected by cardiac puncture. After incubation overnight at 4 °C, the clotted blood cells were removed by centrifugation at 1500 *g* for 10 min. The crude antiserum was frozen in liquid N_2 and stored at −80 °C in 0.04% (w/v) NaN_3. For immunoblotting, the anti-(banana PEPC) IgG was affinity-purified against 25 μg of purified banana PEPC as previously described [19].

Immunotitration of PEPC activity

Immunoremoval of enzyme activity was tested by mixing 0.03 unit of purified PEPC with 25 mM Hepes/NaOH (pH 7.5), containing 0.1 mg/ml BSA, 10% (v/v) glycerol, 1 mM DTT, and various amounts of rabbit anti-(banana PEPC) immune serum, or preimmune serum, diluted into PBS (total volume 0.1 ml). The mixture was incubated for 1 h at 30 °C, and then for 1.5 h on ice, prior to centrifuging for 5 min at 16000 *g* in an Eppendorf microcentrifuge. Residual PEPC activity in the supernatant was determined as described above. Each assay was corrected for contaminating NADH oxidase activity (present in the rabbit immune and preimmune serum) by omitting PEP from the PEPC reaction mixture.

Electrophoresis and immunoblotting

SDS/PAGE was performed according to Laemmli [20] using the Bio-Rad mini-gel apparatus. The final acrylamide monomer concentration in the 0.75-mm-thick slab gels was 10% (w/v) for the separating gel and 4% (w/v) for the stacking gel. Prior to SDS/PAGE, all samples were incubated in the presence of 1% (w/v) SDS and 100 mM DTT for 2 min at 100 °C. Gels were run at a constant voltage of 200 V, applied for 45 min. For the determination of subunit molecular mass by SDS/PAGE, a plot of relative mobility versus log[molecular mass (kDa)] was constructed with the following standard proteins: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa) and ovalbumin (45 kDa). Glycoprotein staining of SDS/PAGE gels was performed using a periodic acid–Schiff procedure [21].

Non-denaturing PAGE was performed using the highly porous SDS/PAGE system of Doucet et al. [22], except that: (i) SDS was omitted from running, sample and gel buffers, and (ii) 20% (v/v) glycerol and 10% (v/v) ethylene glycol were included in both stacking and separating gels. The final concentration of acrylamide was 7% (w/v) in the separating gel and 4% (w/v) in the stacking gel. Gels were pre-cooled to 4 °C before use and were maintained at this temperature during electrophoresis at 200 V for 2 h. Gels were either stained for protein with Coomassie Blue R-250, incubated in a PEPC activity stain, or immunoblotted using anti-(maize leaf PEPC) immune serum as described below. To detect PEPC activity, a lane was incubated for 10 min at room temperature in 100 mM Tris/HCl (pH 8.0) containing 1 mM EDTA, 20 mM $MgCl_2$, 30 mM $NaHCO_3$, 10 mM PEP and 3 mg/ml Fast Violet B. Following staining for PEPC activity gels were fixed with 5% (v/v) acetic acid, and stored in Milli-Q purified water. For estimation of native molecular masses via non-denaturing PAGE, a plot of relative mobility versus log(molecular mass) was constructed using the following protein standards: thyroglobulin (669 kDa), amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (134 and 67 kDa).

For second dimension PAGE, Coomassie Blue-stained PEPC was excised from a native gel and incubated in 200 μl of 62 mM

Table 1 Purification of PEPC from 900 g of ripe banana fruit

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	1840	4674*	949	0.20	1	100
Batchwise DE-52	1240	1290*	574	0.45	2.3	61
Minitan concentration	225	891*	536	0.60	3.0	57
Q-Sepharose	52	105*	495	4.7	24	52
Protein Pak Phenyl-5PW	9	34*	359	10.6	53	38
Chelating Sepharose	5.3	9.0*	189	21.0	105	20
Superose 6 'Prep grade'‡	1.5	3.2*	81	25.3	127	9
		2.5†		32.3		

* Protein determined with the Coomassie Blue G-250 dye-binding assay according to the method of Bollag and Edelstein [28].
† Protein determined with the Pierce bicinchoninic acid reagent according to the method of Hill and Straka [29].
‡ Concentrated pooled fractions.

Tris/HCl (pH 6.8) containing 10% (v/v) glycerol, 5% (v/v) methanol and 2% (w/v) SDS for 2 h at 50 °C with changes of buffer every 30 min. After equilibration in SDS, the gel slice was subjected to SDS/PAGE according to Doucet et al. [22], except that the acrylamide concentration of the separating gel was 7.5% (w/v), and gel thickness was 1 mm.

Immunoblotting was performed as described previously [19], with the exception that poly(vinylidene difluoride) membranes were used in place of nitrocellulose. Antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody [19]. Phosphatase staining was for 10–15 min at 30 °C. Immunological specificities were confirmed by performing immunoblots in which rabbit preimmune serum was substituted for the anti-(maize leaf PEPC) immune serum or affinity-purified anti-(banana fruit PEPC) IgG. Immunoreactive polypeptides were quantified using an LKB Ultroscan XL Enhanced Laser Densitometer.

Determination of native molecular mass via gel-filtration HPLC

This was done at 25 °C using a calibrated Shodex KW-804 gel-filtration column (0.8 cm × 15 cm; exclusion limit 700 kDa) that was pre-equilibrated in buffer H and connected to a Waters 625 HPLC system equipped with a model 486 absorbance detector and a U6K injector. The Waters Baseline and Powerline softwares were used to control the system and for data acquisition and processing. Purified PEPC (100 μg at 500 μg/ml) was chromatographed at a flow rate of 0.5 ml/min and 0.25 ml fractions were assayed for PEPC activity and A_{280}. The native molecular mass was determined from a plot of K_D (partition coefficient) versus log(molecular mass) for the following protein standards: thyroglobulin (669 kDa), apoferritin (443 kDa), catalase (232 kDa), aldolase (158 kDa) and ovalbumin (43 kDa).

Peptide mapping by CNBr cleavage and N-terminal sequencing

Polypeptides were excised individually from an SDS/PAGE mini-gel and cleaved *in situ* with CNBr. The degradation products were analysed on an SDS/14%-PAGE mini-gel according to the method of Plaxton and Moorhead [23]. Following SDS/PAGE, the gel was stained with silver [24].

For N-terminal sequencing, the subunits of banana PEPC were separated by SDS/PAGE as described above, electroblotted on to a poly(vinylidene difluoride) membrane and stained for protein with Coomassie Blue R-250 as described by Matsudaira [25]. Protein staining bands corresponding to the PEPC subunits were excised individually. N-terminal sequencing of each subunit was attempted using an Applied Biosystems model 470A gas-phase sequenator and 120 phenylthiohydantoin amino acid analyser.

Other methods

Extraction of banana fruit under denaturing conditions was performed according to Wu and Wang [26]. This procedure involves tissue homogenization in 10% (w/v) trichloroacetic acid, followed by resolubilization of precipitated proteins in SDS/PAGE sample buffer. This effectively eliminates potential protease activity during tissue extraction and subsequent sample processing [26].

Protein concentration was determined by the dye-binding method of Bradford [27] as modified by Bollag and Edelstein [28], or by the bicinchoninic acid method of Hill and Straka [29]. Bovine γ-globulin was used as the protein standard.

RESULTS

Activities of PK and PEPC during ripening of bananas

The extractable activities of PK and PEPC were monitored at 2 day intervals over the 11 day period following treatment of green, preclimacteric banana fruit with ethylene gas. In agreement with an earlier report [13] no change in the maximal catalytic activity of either enzyme occurred during the ripening time course (data not shown). The average maximal activity of banana fruit PK and PEPC were determined to be 0.93 ± 0.03 and 1.09 ± 0.03 units/g fresh wt., respectively ($n = 18$ each). These values are in the same range as those previously reported by Ball and co-workers [13] for the banana fruit PK and PEPC.

Purification of PEPC from banana fruit

As shown in Table 1, PEPC was purified 127-fold to a final specific activity of 32 units/mg, and an overall recovery of about 9%. Utilization of unbuffered Tris in the homogenization buffer prevented acidification of the extract, and resulted in a clarified homogenate of pH 7.3. The initial batchwise DE-52 fractionation step was necessitated by the high levels of gelatin and pectin present in banana fruit. These substances caused the crude extract to gel within 30 min at 4 °C and thus precluded the initial application of more conventional methods of protein fraction-

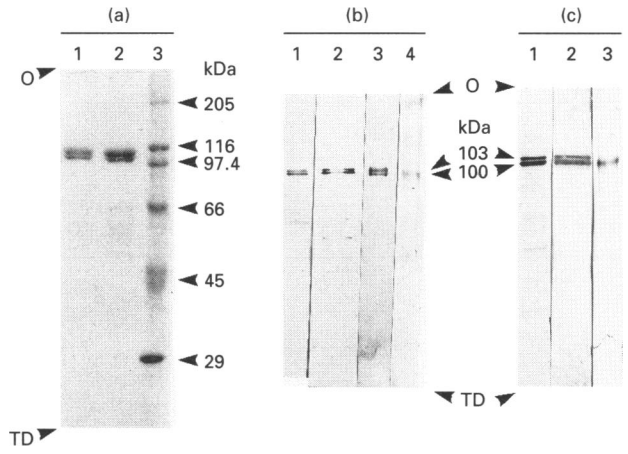

Figure 1 SDS/PAGE and immunoblot analysis of banana fruit and maize leaf PEPCs

(**a**) SDS/PAGE (10% separating gel) of purified banana PEPC. Lanes 1 and 2 contain 0.75 μg of the peak PEPC activity fraction and the pooled peak fractions, respectively, from the final purification step (Superose 6 'Prep grade' FPLC). Lane 3 contains 3 μg of various molecular-mass standards. Protein staining was performed with Coomassie Blue R-250. (**b**) Immunoblot analysis was performed using affinity-purified rabbit anti-(banana PEPC) IgG as described in the Experimental section. Lanes 1 and 2 each contain 0.5 μg of protein from extracts prepared from unripe and ripe banana fruits respectively; lane 3 contains 10 ng of the purified banana PEPC; lane 4 contains 10 ng of partially purified maize leaf PEPC (Sigma, specific activity 3.8 units · mg of protein^{-1}). (**c**) Immunoblot analysis was performed using rabbit anti-(maize leaf PEPC) immune serum as described in the Experimental section. Lane 1 contains 0.5 μg of protein of a crude extract from a ripe banana that had been prepared according to Wu and Wang [26] so as to eliminate potential protease activity during enzyme extraction and sample processing. Lane 2 contains 50 ng of the purified banana PEPC; lane 3 contains 10 ng of partially purified maize leaf PEPC (Sigma). Abbreviations: O, origin; TD, tracking dye front.

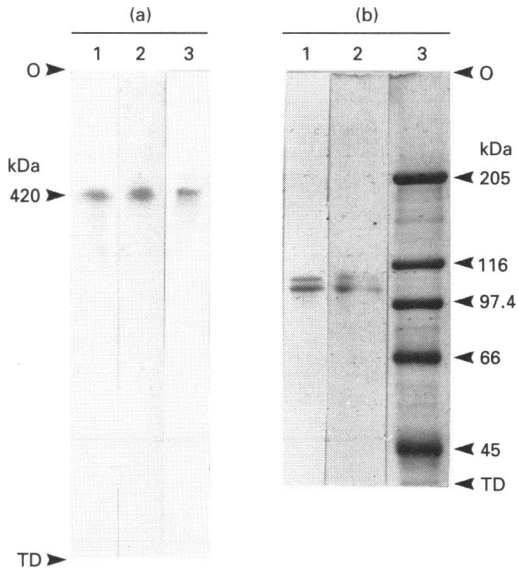

Figure 2 Non-denaturing PAGE followed by SDS/PAGE of purified banana fruit PEPC

(**a**) Non-denaturing PAGE (7% separating gel) of purified banana PEPC. The gel was stained for protein with Coomassie Blue R-250 (lane 1) or for PEPC activity (lane 2) as described in the Experimental section. Lane 3 is an immunoblot of 25 ng of the final preparation that was probed with anti-(maize leaf PEPC) immune serum. The protein-stained lane and the lane used to detect PEPC activity each contained 2 μg of protein. (**b**) SDS/PAGE (7.5% separating gel) of the protein-staining band shown in (**a**) that had been excised from the non-denaturing gel and equilibrated with SDS, and immunoblotted using anti-(maize PEPC) immune serum (lane 1), or stained for protein (lane 2). Lane 3 contains 5 μg of various molecular-mass standards. Lanes 2 and 3 were stained with Coomassie Blue R-250. Abbreviations: O, origin; TD, tracking dye front.

ation such as poly(ethylene) glycol or $(NH_4)_2SO_4$ precipitation, or column chromatography.

Physical and immunological properties

Gel electrophoresis

Denaturation, followed by SDS/PAGE of the final preparation, resolved two equal-intensity protein-staining bands of approximately 103 and 100 kDa (Figure 1a, lane 2). An identical result was obtained when the peak PEPC activity fraction obtained following the final purification step (Superose 6 gel-filtration FPLC) was analysed by SDS/PAGE (Figure 1a, lane 1). Non-denaturing PAGE of the final preparation using separating gel acrylamide concentrations of 5.0% or 7.0% (w/v) generated a well-resolved single protein-staining band of about 420 kDa which co-migrated with PEPC activity and cross-reacted strongly with anti-(maize leaf PEPC) immune serum. Representative results are seen in Figure 2(a) for the 7.0% (w/v) gel. When the 420 kDa protein-staining band present following non-denaturing PAGE was excised, equilibrated with SDS and subjected to SDS/PAGE, the 103 kDa and 100 kDa polypeptides were resolved and: (i) cross-reacted strongly with anti-(maize leaf PEPC) immune serum (Figure 2b, lane 1); and (ii) stained for protein with similar intensities (Figure 2b, lane 2).

Following SDS/PAGE of 10 μg of purified banana PEPC, the 103 and 100 kDa polypeptides could not be detected by periodic acid–Schiff staining, indicating that protein-bound carbohydrate was absent (results not shown).

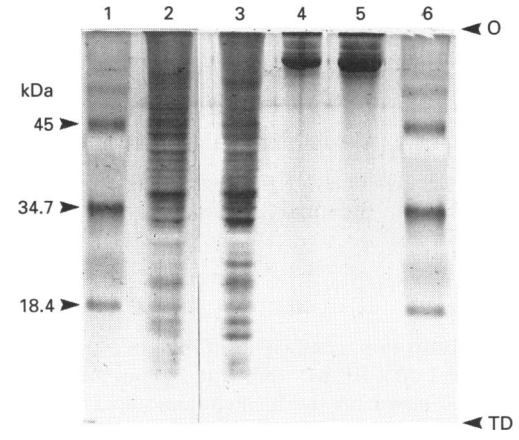

Figure 3 Electrophoretic patterns of CNBr-cleavage fragments of the 103 and 100 kDa subunits of banana fruit PEPC

CNBr-cleavage fragments were prepared from gel slices containing 10 μg of the 103 (lane 2) and 100 kDa (lane 3) polypeptides and analysed on an SDS/14%-PAGE mini-gel as previously described [23]. Lanes 4 and 5 contain 10 μg of the 103 and 100 kDa polypeptides respectively, that were incubated in the absence of CNBr. Lanes 1 and 6 contain 4 μg of various molecular-mass standards. The gel was stained with silver according to the method of Wray et al. [24]. Abbreviations: O, origin; TD, tracking dye front.

Table 2 Effects of glycerol, pH and various metabolites on $V_{max.}$, h and K_m(PEP) or $S_{0.5}$(PEP) of banana fruit PEPC

The standard spectrophotometric assay [±10% (v/v) glycerol] was used except that the PEP concentration was varied and effectors were added at the concentrations shown in the table. Hill coefficients (h) were equivalent to 1.0, except where the value for h is indicated in parentheses. Abbreviation: n.d., not determined.

	pH 7.0				pH 8.0			
	$V_{max.}$ (units/mg of protein)		K_m(PEP) (mM)		$V_{max.}$ (units/mg of protein)		K_m(PEP) (mM)	
	−Glycerol	+Glycerol	−Glycerol	+Glycerol	−Glycerol	+Glycerol	−Glycerol	+Glycerol
Control	16	19	0.140	0.041	31	31	0.078	0.040
1 mM Glc 6-*P*	28	26	0.030	0.011	n.d.	n.d.	n.d.	n.d.
1 mM Gly 3-*P*	29	32	0.018	0.017	n.d.	n.d.	n.d.	n.d.
50 μM Malate	16	19	1.4* (2.1)	0.94	n.d.	n.d.	n.d.	n.d.

*This value represents an $S_{0.5}$(PEP) as $h >> 1.0$.

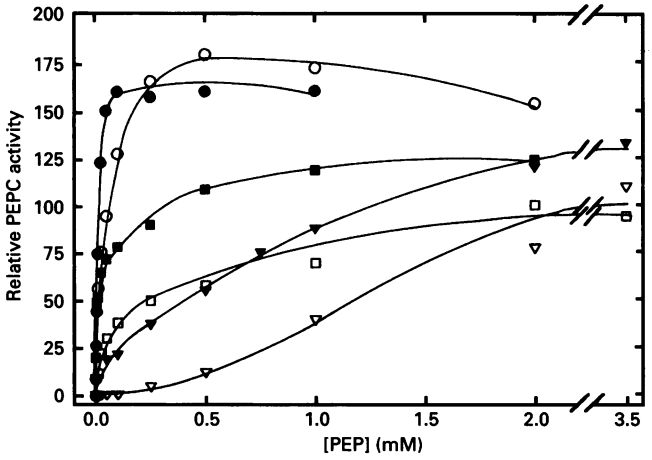

Figure 4 Effect of glycerol, Glc 6-*P*, and malate on the PEP saturation kinetics of banana fruit PEPC

Assays were conducted at pH 7.0 in the presence (■,●,▼) and absence (□,○,▽) of 10% (v/v) glycerol. The designations are: (■,□), control; (●,○), +1 mM Glc 6-*P*; (▼,▽), +50 μM malate.

Table 3 Effect of various metabolites on the activity of banana PEPC

Assays were conducted at pH 7 or 8 in the presence of 10% (v/v) glycerol using a subsaturating (75 μM) concentration of PEP. Enzyme activity in the presence of effectors is expressed relative to the respective control set at 100. In all instances the final effector concentration was 2 mM.

	Relative activity	
Addition	pH 7.0	pH 8.0
Glc 1-*P*	227	127
Glc 6-*P*	245	125
Fru 1-*P*	231	125
Fru 6-*P*	253	123
Gly 3-*P*	247	127
Malate	9	76
Succinate	19	79
Aspartate	4	100
Glutamate	9	94
MgATP	54	37

Determination of native molecular mass via gel-filtration HPLC

The native molecular mass of the enzyme as estimated by gel-filtration HPLC of the final preparation was 425±23 kDa (mean±S.E.M., $n = 3$).

Absorption coefficient

The molar absorption coefficient of banana PEPC was determined to be 1.50×10^5 $M^{-1} \cdot cm^{-1}$ at 280 nm ($A^{0.1\%}_{1\,cm,280}$ 0.355). This value was based on the bicinchoninic acid determination of protein concentration [29] and calculated by assuming a native molecular mass of 425 kDa.

Peptide mapping and N-terminal sequencing

The structural relationship between the 103 and 100 kDa polypeptides was investigated by peptide mapping of their CNBr cleavage fragments and N-terminal sequencing. As shown in Figure 3, the CNBr cleavage patterns of the two polypeptides were similar, but not identical. These results indicate that the banana PEPC subunits are homologous, but non-identical polypeptides. N-terminal analysis was attempted, but it was revealed that both the 103 and 100 kDa subunits were blocked at their N-termini.

Immunological characterization

Increasing amounts of rabbit anti-(banana PEPC) immune serum immunoprecipitated up to 100% of the activity of the purified banana PEPC; complete immunoremoval of activity occurred at about 70 μl of immune serum per unit PEPC activity (results not shown). By contrast, pre-immune serum had no effect on the activity of banana PEPC (results not shown).

Rabbit anti-(banana PEPC) immune serum was affinity-purified using 25 μg of homogeneous PEPC as previously described [19]. The affinity-purified anti-(banana PEPC) IgG could detect as little as 1 ng of homogeneous non-denatured banana PEPC that had been dot blotted on to nitrocellulose (results not shown). Analysis of this antibody preparation demonstrated that it is monospecific for the two polypeptides present in the purified PEPC, as only these polypeptides showed a significant cross-reaction with the anti-(banana PEPC) IgG when a crude extract prepared from unripe or ripe bananas was blotted (Figure 1b, lanes 1 and 2). Immunoreaction of both the 103 and 100 kDa polypeptides is also seen with nanogram quantities of the

Table 4 Kinetic constants for several effectors of banana PEPC

Assays were conducted at pH 7 or 8 in the absence and/or presence of 10% (v/v) glycerol using a subsaturating (75 μM) concentration of PEP. Abbreviations: n.d., not determined; n.e., no effect on enzymic activity under these assay conditions.

	pH 7.0				pH 8.0	
	I_{50} (mM)		K_a (mM)		I_{50} (mM)	K_a (mM)
Effector	−Glycerol	+Glycerol	−Glycerol	+Glycerol	+Glycerol	+Glycerol
Glc 1-*P*	–	–	0.24	0.18	–	0.29
Glc 6-*P*	–	–	0.15	0.020	–	0.037
+50 μM Malate	–	–	0.39	0.068	–	n.e.
Fru 1-*P*	–	–	1.3	0.068	–	0.21
Fru 6-*P*	–	–	1.2	0.067	–	0.24
Gly 3-*P*	–	–	0.26	0.15	–	0.20
Malate	0.015	0.011	–	–	8.0	–
+1 mM Glc 6-*P*	0.12	1.10	–	–	n.d.	–
Succinate	0.68	0.42	–	–	6.3	–
Aspartate	0.054	0.070	–	–	n.e.	–
Glutamate	0.34	0.34	–	–	n.e.	–
MgATP	1.2	1.3	–	–	1.7	–

homogeneous PEPC (Figure 1b, lane 3). Since both polypeptides appear to be present in crude banana extracts that were prepared under totally denaturing conditions (Figure 1c, lane 1), this indicates that the 100 kDa polypeptide did not arise as a result of proteolysis of the 103 kDa polypeptide during banana extraction and subsequent PEPC purification. An immunoblot of a commercial preparation of maize leaf PEPC revealed a single immunoreactive polypeptide migrating with a molecular mass of about 100 kDa when probed with the affinity-purified anti-(banana PEPC)-IgG (Figure 1b, lane 4). Similarly, when an immunoblot of a crude banana extract or the purified banana PEPC was probed with anti-(maize leaf PEPC) immune serum, the 103 and 100 kDa immunoreactive polypeptides were obtained and stained with approximately equal intensities (Figure 1c, lanes 1 and 2).

Kinetic properties

Linearity of PEPC activity with respect to time

Following dilution in the assay medium, banana PEPC demonstrated a time-dependent reduction in activity at both pH 7.0 and 8.0. Although PEPC activity remained linear with respect to time over the initial 0.5 min following dilution of the enzyme in the pH 7.0 assay medium, enzyme activity typically decreased by up to 50% over the subsequent 1 min assay interval. Enzyme kinetic studies at pH 7.0 therefore employed an assay period of 0.5 min. At pH 8.0, the PEPC reaction rate remained linear with respect to time for at least 1 min, although longer incubation periods provoked a slight reduction in activity. Thus, all kinetic studies at pH 8.0 were done using a 1 min assay period.

Effect of pH

The enzyme exhibited a broad pH/activity profile similar to that of other PEPCs [1,4,6–8,30,31], with a maximum occurring between pH 7.5 and 9.0 (results not shown). PEPC activity at pH 7.0 was approximately 50% of that at pH 8.0 (Table 2). The addition of 10% (v/v) glycerol to the reaction mixture did not alter the pH/activity profile for the banana PEPC (results not shown).

Cation requirements

As is the case with other PEPCs [1,4], the banana enzyme showed an absolute dependence for a bivalent cation. At pH 8.0, Mn^{2+} (12 mM, added as $MnCl_2$) yielded the same $V_{max.}$ value achieved with saturating Mg^{2+}. Moreover, an identical K_m value of 0.1 mM was obtained for Mg^{2+} and Mn^{2+} at pH 8.0.

Substrate saturation kinetics

Table 2 summarizes $V_{max.}$, apparent K_m(PEP) or $S_{0.5}$(PEP), and Hill coefficient (h) values at pH 7.0 and 8.0 in the presence and absence of 10% (v/v) glycerol. In common with most other plant PEPCs [1,4,6–8,30], the banana enzyme displayed hyperbolic PEP saturation kinetics in the absence of added effectors (Table 2 and Figure 4). Decreasing assay pH to 7.0 from 8.0 increased the enzyme's K_m(PEP) by about 2-fold. The inclusion of 10% (v/v) glycerol in the assay medium decreased the enzyme's K_m(PEP) by 50 and 70% at pH 8.0 and 7.0 respectively, and served to negate the pH-dependent differences in K_m(PEP) (Table 2). Other authors have cited stabilization of the quaternary structure of PEPC, due to exclusion of solvent molecules, as the explanation for the favourable effect of glycerol on the affinity of PEPC for PEP [31]. A $K_m(HCO_3^-)$ of 64 μM was obtained at pH 8.0 in the absence of added glycerol.

Metabolite effects

A wide variety of compounds was tested as possible effectors of PEPC at pH 7.0 and 8.0 with subsaturating concentrations of PEP (75 μM) in the presence of 10% (v/v) glycerol. The following substances, at concentrations of up to 5 mM, had no effect on PEPC activity (±10% of control rate) at either pH 7.0 or 8.0: potassium phosphate, 3-phosphoglycerate, 2-phosphoglycerate, isocitrate, AMP, dihydroxyacetone phosphate, NAD^+, fructose 1,6-bisphosphate, acetyl-CoA, 2-oxoglutarate, L-arginine, L-asparagine, L-phenylalanine, L-glutamine, L-glycine, L-alanine, and L-leucine. Table 3 lists those compounds which were found to activate or inhibit the activity of the purified enzyme. As shown in Table 3, the enzyme displayed pH-dependent modulation by several metabolites such that they were generally far more

effective at pH 7.0 than pH 8.0. Similar observations have been reported for the PEPC from various sources including soybean root nodules [6], a green alga [7], cotyledons of germinated castor seeds [8], avocado fruit [30], and maize leaves [31].

Activators

At pH 7.0, the banana PEPC was markedly activated by several hexose-monophosphates and glycerol 3-phosphate (Gly 3-*P*) (Table 3). At 1 mM, both glucose 6-phosphate (Glc 6-*P*) and Gly 3-*P* significantly decreased the enzyme's K_m(PEP) (Table 2 and Figure 4). Synergistic or additive effects of activators at pH 7.0 were not observed, suggesting that they are all interacting at a common allosteric site. The addition of 50 μM malate increased PEPC's K_a(Glc 6-*P*) by over 2-fold, whereas the presence of 10% (v/v) glycerol markedly increased the affinity of the enzyme for the various activators at pH 7.0 (Table 4). Increasing the pH to 8.0 from 7.0 not only decreased the extent of activation of the enzyme by the four hexose-phosphates and Gly 3-*P* (Table 3), but also increased the K_a values for these activators by up to 3-fold (Table 4). Although the five activators exerted a similar fold-activation of PEPC activity (Table 3), the enzyme's K_a(Glc 6-*P*) was consistently lower than the value obtained with any of the other activators (Table 4).

Inhibitors

At pH 7.0, the banana PEPC was potently inhibited by malate, succinate, aspartate and glutamate (Tables 3 and 4). Malate yielded an extremely low I_{50} of 11–15 μM at pH 7.0 (Table 4), with near-complete inhibition of PEPC activity occurring at a concentration of 2 mM (Table 3). In the absence of glycerol, 50 μM malate promoted positive cooperativity with respect to the binding of PEP and increased the K_m(PEP) by 10-fold (Table 2 and Figure 4). The inclusion of 10% (v/v) glycerol in the pH 7.0 assay medium slightly lowered the I_{50}(malate) and served to abolish the malate-induced positive cooperativity with respect to the binding of PEP (Tables 2 and 4, Figure 4). The addition of 1 mM Glc 6-*P* at pH 7.0 increased the I_{50}(malate) by 10- and 100-fold in the absence and presence of 10% (v/v) glycerol respectively (Table 4). Increasing the assay pH to 8.0 from 7.0 greatly decreased or eliminated the enzyme's inhibition by malate, succinate, aspartate and glutamate (Tables 3 and 4). Inhibition of banana PEPC by MgATP was also observed (Table 3). However, MgATP was a somewhat weaker inhibitor relative to malate, succinate, aspartate or glutamate (Tables 3 and 4). Moreover, unlike the other effectors, the enzyme's susceptibility to inhibition by MgATP was comparable at pH 7.0 and 8.0 (Tables 3 and 4).

DISCUSSION

The extractable maximal activities of banana fruit PEPC and PK were substantial, equivalent, and remained uniform during the ripening time course. This data suggests that both enzymes could effectively compete for a common pool of cytosolic PEP and that the stimulation of PEP utilization which appears to be the initial response of glycolysis at the respiratory climacteric [12,13] arises from a fine control activation of pre-existing enzymes, rather than via *de novo* synthesis of PK_c and/or PEPC. Despite the relative abundance of PEPC in banana fruit, a recent study analysing fluxes of carbohydrate metabolism in climacteric bananas has indicated that the rate of dark CO_2 fixation (presumably representing *in vivo* PEPC activity) is negligible compared to the tissue's overall respiratory flux [32]. This result, however, was based upon analysing the incorporation of exogenous $^{14}CO_2$ by cores of climacteric fruit. Thus, the calculation of the rate of dark CO_2 fixation by climacteric bananas could potentially have been underestimated owing to refixation of respired (non-radioactive) CO_2 by PEPC. Moreover, as Hill and ap Rees [32] restricted their analysis to climacteric fruit, the possibility that PEPC makes an important contribution to the carbon flux of post-climacteric bananas cannot be discounted.

PEPC purification and subunit composition

The PEPC of ripened bananas was purified 127-fold to a final specific activity of 32 units/mg (Table 1). This final specific activity compares favourably to the values reported for homogenous PEPCs from various C_3- and C_4-metabolizing plant tissues [33–36]. Similar to most other PEPCs, the native molecular mass of the purified enzyme was determined by gel-filtration HPLC to be about 425 kDa. However, two polypeptides of 103 and 100 kDa, which stained for protein with equal intensity, were observed when the purified banana PEPC was subjected to SDS/PAGE (Figure 1a, lane 2). We believe that both polypeptides are associated with native banana PEPC for the following reasons: (i) the 420 kDa protein-staining band obtained following non-denaturing PAGE co-migrated with PEPC activity and still produced the 103 and 100 kDa polypeptides upon SDS/PAGE (Figure 2); (ii) both polypeptides cross-react strongly with anti-(maize leaf PEPC) immune serum (Figure 1c, lanes 1 and 2); (iii) the two protein staining bands co-eluted following Superose 6 gel-filtration FPLC (Figure 1a, lane 1); and (iv) the respective patterns of peptide fragments produced after partial digestion with CNBr are quite comparable, as analysed by SDS/PAGE (Figure 3). The subunit heterogeneity of the banana PEPC does not appear to have arisen following tissue extraction by the action of endogenous proteases (Figure 1c, lane 1). We cannot exclude the possibility that our final enzyme preparation consists of an equivalent mixture of 412 and 400 kDa homotetrameric PEPC isoforms. However, (i) immunoblotting of crude extracts revealed that the equivalent ratio of the 103 and 100 kDa antigenic polypeptides remains uniform during the transformation of unripe (metabolically dormant) fruit into ripe (metabolically active) fruit (Figure 1b, lanes 1 and 2), and (ii) kinetic studies are consistent with the presence of a single active PEPC species in the final preparation. These results suggest that unlike most homotetrameric PEPCs analysed to date, the banana PEPC exists predominantly as a heterotetramer composed of equivalent amounts of the 103 kDa (α-subunit) and 100 kDa (β-subunit) polypeptides. Similarly, immunoblot analyses have indicated that the PEPC from germinated *Ricinus communis* seeds and guard cells of *Vicia fabia* leaves could contain equal proportions of 108 and 103 kDa, and 112 and 110 kDa subunits respectively [37,38]. However, to the best of our knowledge, the present study represents the first purification to homogeneity of an apparent $\alpha_2\beta_2$ heterotetrameric PEPC from any source. Additional studies are required to establish whether the subunit heterogeneity of the banana PEPC arises as a result of the expression of separate genes or is derived from a single gene by differential post-translational modification.

Immunological properties

Rabbit anti-(banana PEPC) immune serum immunoprecipitated up to 100% of the activity of the purified banana PEPC. Monospecificity of the antibody preparation for PEPC is indi-

cated by the observation that only the α- and β-subunits of banana PEPC demonstrated any significant cross-reaction when immunoblots of crude extracts from unripe or ripened fruit were probed with the affinity-purified anti-(banana PEPC) IgG (Figure 1b, lanes 1 and 2). Antibodies to banana PEPC cross-reacted strongly with maize PEPC (Figure 1b, lane 4) and vice versa (Figure 1c, lanes 1 and 2; Figure 2a, lane 3; Figure 2b, lane 1). Similarly, PEPCs from soybean root nodules and castor oil seeds are recognized by the same anti-(maize leaf PEPC) immune serum as was used in the present study [6,8,37]. These immunological data indicate a high degree of structural similarity between the C_4- and C_3-type PEPCs of higher plants. By contrast, the anti-(banana or maize PEPC) IgG fails to recognize a purified PEPC from the green alga *Selenastrum minutum* (R. H. Dunford, W. C. Plaxton and D. H. Turpin, unpublished work).

Effect of dilution on banana PEPC

The activity of banana PEPC was stable when the final concentrated preparation was stored at −80 °C for several months, or on ice for several hours. However, dilution in the assay medium provoked a rapid loss of catalytic activity. Protein concentration has been demonstrated to influence the aggregation state and activity of PEPC from C_4 and CAM leaves [31,34,35]. Hence, the dilution-dependent reduction in PEPC activity may arise from dissociation of the active native PEPC heterotetramer into inactive lower-molecular-mass forms. This hypothesis is corroborated by the striking effect that the addition of glycerol to the PEPC assay medium has on the enzyme's kinetic and regulatory properties. The presence of glycerol (10%, v/v), a compound that can promote aggregation of proteins [39] including maize leaf PEPC [31], was found to: (i) decrease the K_m(PEP) by up to 3-fold and eliminate positive cooperativity with respect to PEP binding in the presence of 50 μM malate (Table 2 and Figure 4); (ii) reduce the K_a values for hexose-monophosphates and Gly 3-*P* by up to 7.5-fold; and (iii) greatly amplify the ability of Glc-6-*P* to relieve malate inhibition at pH 7.0 (Table 4).

Metabolite effects on banana PEPC

Our results demonstrate that the PEPC of ripened bananas is regulated by metabolites involved in carbohydrate and nitrogen metabolism in a pH-dependent fashion (Tables 3 and 4). PEPC was markedly activated by hexose-monophosphates and potently inhibited by malate, succinate, aspartate and glutamate at pH 7.0, whereas the response of the enzyme to these effectors was considerably diminished or totally eliminated at pH 8.0 (Tables 3 and 4). At pH 7.0, the K_a or I_{50} values for these compounds are well within their probable physiological concentration range, suggesting that these metabolites may be important regulators of PEPC *in vivo*, especially at pH values close to neutrality. Potent inhibition of plant PEPC by aspartate and glutamate is not widespread, but has been reported for the enzyme from soybean and lupin root nodules [6,40], the green alga *S. minutum* [7], and cotyledons of germinated castor seeds [8]. By contrast, activation by Glc 6-*P* and inhibition by malate appear to be a general feature of most plant PEPCs examined to date [1,4,6–8,30,31]. However, the banana PEPC's I_{50}(malate) of about 11 μM is orders of magnitude lower than the value reported for the enzyme from most other sources. A notable exception is the PEPC from avocado fruit which exhibited an equivalent I_{50}(malate) of 10 μM at pH 6.8 [30]. Exceptional sensitivity to inhibition by malate may represent a general feature of fruit PEPCs.

Metabolite regulation of PEPC in relation to banana fruit metabolism

Hill and ap Rees [32] have demonstrated that starch-derived hexose-monophosphates are probably the major form of carbon exported from the amyloplast of ripening banana fruit. The activation of PEPC by hexose-monophosphates could therefore coordinate the rate of starch mobilization with the production of dicarboxylic acids via PEP carboxylation. Activation of PEPC should also arise in response to cytosolic alkalinization owing to direct pH effects on its activity as well as the enzyme's desensitization to inhibitory metabolites. This is consistent with the proposed pH-stat function of fruit PEPC [30]. Continued PEPC activity should eventually lower cytosolic pH to a point where the enzyme becomes increasingly susceptible to feedback inhibition by amino acids and tricarboxylic acid cycle intermediates. However, the regulatory properties of the banana PEPC indicate that this enzyme may play an additional vital function in coordinating carbon and nitrogen metabolism in this tissue. Significant rates of *de novo* protein synthesis have been demonstrated in ripening banana fruit [41]. PEPC may therefore fulfil a key anaplerotic role by replenishing dicarboxylic acids consumed through transamination reactions. In particular, the inhibition of the enzyme by aspartate and glutamate provides a tight feedback control which could closely balance overall PEPC activity with the production of carbon skeletons (i.e. oxaloacetate and 2-oxoglutarate) required for NH_4^+ assimilation and transamination reactions in ripening bananas. Recent studies indicating activation of the wheat leaf enzyme by protein kinase-mediated phosphorylation in response to nitrate feeding of nitrogen-deprived seedlings [42] raise the possibility for additional mechanisms associated with the metabolic regulation of PEPC in ripening fruit.

This work was supported by The Natural Sciences and Engineering Research Council of Canada (NSERC). We are grateful to Dr. Raymond Chollet for the gift of antibodies against maize leaf PEPC, and to Dr. Stephen Duff for helpful discussions. We also wish to thank Dr. Rosario Munoz-Clares for advice concerning non-denaturing PAGE of PEPC.

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Received 18 August 1994/12 December 1994; accepted 21 December 1994